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Acetylcholinesterase Enzyme Activity in Brain Tissue of Brook Trout (Salvelinus fontinalis) Collected After the 1988 Hemlock Looper Control Program in Newfoundland

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ACETYLCHOLINESTERASE ENZYME ACTIVITY IN BRAIN TISSUE OF BROOK TROUT (SALVELINUS FONTINALIS) COLLECTED AFTER THE 1988 HEMLOCK LOOPER CONTROL PROGRAM IN NEWFOUNDLAND

by

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ABSTRACT

Fancey, L. L., J. F. Payne, J. W. Kiceniuk, and S. Ray. 1990. Acetylcholinesterase enzyme activity in brain tissue of brook trout (Salvelinus fontinalis) collected after the 1988 hemlock looper control program in Newfoundland. Can. Tech. Rep. Fish. Aquat. Sci. 1763: iv + 11 p.

During the 1988 hemlock looper control program carried out by the Newfoundland and Labrador Dept. of Forestry, a field monitoring program was undertaken to compare brain acetylcholinesterase (AChE) enzyme activities of brook trout (Salvelinus fontinalis) collected from a stream in a fenitrothion spray block, with enzyme activities in trout collected from a similar size stream in an unsprayed area.

Residues of fenitrothion could not be detected in the treated stream. However, there was a small but statistically significant, reduction in enzyme activity (9.2%) in the trout collected after the second application of fenitrothion in the spray block compared to trout from the control site. Since this field trial represented a worse case scenario of two spray applications over a small stream with no buffer zones present spraying practices in Newfoundland should have negligible toxicological effects on fish.

RÉSUMÉ

Fancey, L. L., J. F. Payne, J. W. Kiceniuk, and S. Ray. 1990. Acetylcholinesterase enzyme activity in brain tissue of brook trout (Salvelinus fontinalis) collected after the 1988 hemlock looper control program in Newfoundland. Can. Tech. Rep. Fish. Aquat. Sci. 1763: iv + 11 p.

Au cours du programme de contrôle de l'arpenteuse de la pruche exécuté par le ministère des Forêts et de l'Agriculture de Terre-Neuve et du Labrador en 1988, on avait mis au point un projet de surveillance sur le terrain dont l'objet était de comparer l'activité de l'acétylcholinestérase du cerveau des truites mouchetées (Salvelinus fontinalis) prélevées d'un cours d'eau situé dans une zone d'arrosage au fénithrothion, avec la même activité chez des truites échantillonnées dans un cours d'eau semblable en dehors du secteur arrosé.

On n'a pu déceler aucun résidu de fénithrothion dans le cours d'eau arrosé. Cependant, on a remarqué une petite réduction, quoique importante sur le plan statistique, de l'activité enzymatique (9,2 p. 100), après une deuxième application du fénithrothion dans la zone d'arrosage. La truite du cours d'eau témoin n'a pas enregistré cette réduction. Comme cet essai sur le terrain représente le scénario du pire cas possible, c'est-à-dire deux applications de fénithrothion dans un petit cours d'eau n'ayant aucune zone tampon, on estime que les pratiques d'arrosage actuelles à Terre-Neuve devraient avoir très peu d'effets toxicologiques sur le poisson.

INTRODUCTION

The eastern hemlock looper (Lambdina fiscellaria fiscellaria (Guen)) control program, conducted in 1988 by the Newfoundland and Labrador Department of Forestry, consisted of aerial spray applications of the insecticide fenitrothion (O,O-dimethyl-O-(3-methyl-4-nitrophenyl) phosphorothioate) over areas of infestation mainly on the Great Northern Peninsula in Western Newfoundland. Fenitrothion, an organophosphate insecticide, which has been used in Newfoundland from 1985 to 1988 to control the hemlock looper, acts as an inhibitor of the enzyme acetylcholinesterase (AChE, EC 3.1.1.7). This inhibition results in the accumulation of the neurotransmitter acetylcholine in cholinergic synapses preventing the transmission of nerve impulses.

A field monitoring program was undertaken during June and July, 1988, to address concerns of possible effects of fenitrothion on potentially sensitive fish populations within sprayed areas. Brain AChE activities of brook trout (Salvelinus fontinalis) from a small unbuffered stream within a sprayed area, were compared to those from a similar size stream in an unsprayed or reference area.

Decreased enzyme activities in fish from treated versus reference sites serve to indicate whether fenitrothion or its degradation products have entered a sampling area at concentrations sufficient to cause systemic poisoning. Brain cholinesterase activity measurements have been used in laboratory and field studies to detect organophosphate exposure in fish. (Weiss 1961; Williams and Sova 1966; Holland et al. 1967; Lockhart et al. 1973; Coppage and Matthews 1974; Coppage and Braidech 1976; Zinkl et al. 1987; Fancey et al. 1987). The AChE enzyme technique may be used in monitoring programs as an early warning indicator of fenitrothion toxicity and to help address concerns about the effects of pesticides on fish.

MATERIALS AND METHODS

SPRAY PROGRAM

Fenitrothion was applied aerially to eighteen (18) blocks totalling 45,138 hectares in 1988. Each block was sprayed twice at an application rate of 210 g active ingredient per ha per application. The composition of the operational spray formulation was (by volume) 11% fenitrothion (Folithion® O,O-dimethyl O-(3-methyl-4-nitrophenyl) phosphorothioate) Chemagro Ltd., Ontario, PCP #10776, 40% Cyclosol 63 and 49% insect diluent 585 or common stove oil. (Hubert Crummey, Dept. of Forestry, pers. comm).

Spray block 113 (5,122 ha) sampling site was first sprayed on July 14 between 8:00 PM and 8:54 PM and received a second spray application on July 20 between 8:34 PM and 9:29 PM. All flight lines were completed as indicated in Fig. 1. (John Smith, Dept. of Forestry, pers. comm.)

SAMPLING SITE LOCATIONS AND FISH SAMPLING

Both the sprayed and control sampling sites were located on the Great Northern Peninsula in Newfoundland (Fig. 2).

The sprayed site was a small stream within spray block 113 situated near Flat Pond. The stream was chosen over other larger rivers in the area because of good accessibility for fish collection. Also, this stream was small enough so that the regular buffer zones (Cahill, 1987) generally applied to larger bodies of water, in Newfoundland, were not used. The control site was a similar size stream located 5.7 km north of Eddies Cove West. The control stream was chosen so as to be well away from any sprayed area.

Juvenile brook trout, 10 to 15 cm in length, were collected by electrofishing at both sites. Thirty fish were taken at each sampling time (prespray, June 9; 1st postspray, 72 hr after 1st application, July 17; and 2nd postspray, July 22, 48 hr after the second spray application), at both control and spray sampling locations. Several extra fish were also taken to serve as pooled controls for checking assay reliability on a day to day basis. Fish were frozen on dry ice immediately after collection and transported to the laboratory where they were stored at -60C until analyzed.

WATER SAMPLING AND ANALYSIS

Three replicate, 1 liter, water samples were taken from well separated, randomly chosen, areas within each stream site. Immediately after collection in the field, each sample was transferred to a separatory funnel and individually extracted with 3 x 30 mL 1,2-dichloromethane (Fisher Scientific, HPLC grade, D143). Extracts were separated, collected in amber bottles that had been previously rinsed with hexane and 1,2-dichloromethane, and transported to the laboratory for subsequent analysis for fenithrothion by high performance liquid chromatography (HPLC).

In the laboratory, each extract was reduced in volume and solvent exchanged to methanol in a rotary evaporator, then made to 1 mL with methanol. The extract was then filtered through a 0.45 μm, 13 mm, filter (Supelco) into a 1.8 mL autosampler vial for analysis by HPLC.

The HPLC system consisted of an ISS-100 autosampler, Series 4 pump, and LC-85B spectrophotometric detector and a Model 3600 data station, each from Perkin-Elmer.

Chromatographic separations were done on a poly(styrenedivinylbenzene) column (Hamilton PRP-1, 10 μm, 250 mm x 4.1 mm) with an injection volume of 10 ul. The mobile phase consisted of 25% solvent A, (1:1 acetonitrile in water) and 75% solvent B (acetonitrile), at a flow rate of 2 mL min^{-1}.

Fenitrothion (Mobay Chemical Corp., 0.005 $\mu g\ \mu l^{-1}$) was used as an external

standard and peaks were identified on the basis of retention times. Quantification was done by comparison of the absorbance (269 nm) of the extracts with those of the standards.

The detection limit of fenithrothion was ca. 0.5 ng at a signal to noise ratio of 3:1.

Water samples were collected, after the second spray application, from two bogs approximately two and three kilometers respectively, from the sprayed stream site within spray block 113. Control bog water was taken from an unsprayed bog approximately five kilometers south of Daniel's harbour (Fig. 1). These samples were also extracted in the field and the extracts transported to the laboratory for analysis. Bog water samples were collected to determine residue levels of fenithrothion in stagnant acidic waters as compared to flowing stream water.

Recovery studies were carried out using "brown" water samples collected from two streams within the St. John's, urban area. The water samples were spiked with fenithrothion (Mobay Chemical Corp.) to have a final concentration of 5.95 ng mL^{-1}. Duplicate spiked samples were tested for fenithrothion residues after standing 2 and 24 hr in amber bottles. The control and spiked samples were extracted with 3 x 30 mL of 1,2-dichoromethane and each extract was prepared for analysis by HPLC as described above. The recovery for the 2 and 24 hr samples were 95-98 and 88-94% respectively.

BRAIN ACETYLCHOLINESTERASE ACTIVITY MEASUREMENTS

Fish samples were stored frozen whole at -60°C for approximately seven months before analysis. Brain tissues of fish collected from control and spray areas were individually analyzed for AChE activity. Each fish in a sample was thawed slightly before the whole brain was removed.

Each brain was homogenized in 0.05 M Trizma buffer, pH 8, at a ratio of 1 mL of buffer for each 100 mg of brain tissue (91 mg mL^{-1}). Homogenates were prepared using ten passes of the pestle of a 2 mL Ten Broeck hand tissue grinder.

The homogenate was then centrifuged at 9,000 g for 10 min to separate small fragments of melanin-containing (black) tissue which could interfere with absorbance. The whole homogenate was resuspended with a pasteur pipette and transferred to 1.5 mL Eppendorf centrifuge tubes. No visible pellet remained in the centrifuge tube and the homogenate was considered to be at the ratio of 91 mg mL^{-1}. The homogenates were stored for 1 month at -60°C until analysed for acetylcholinesterase activity (AChE).

Brain AChE activity was assayed by the method of Ellman (1961) as modified by Hill and Fleming (1982) and Hill (1988). All assays were run in duplicate on a Perkin-Elmer recording, scanning spectrophotometer (Coleman 571). Cuvettes were of 1 cm optical path length and assays were conducted at 23°C. Absorbance was read at 405 nm. (see Appendix for assay details).

STATISTICAL ANALYSES OF DATA

Data were tested for normality of distribution (SAS Univariate) and analysis of variance (ANOVA) was used to examine differences. When the analysis indicated a significant difference ($p < 0.05$), means were compared using Duncan's Multiple Range test.

RESULTS

Data were found to be normally distributed. Two way analysis of variance (ANOVA) was first used to examine for effects of treatment (control vs. sprayed streams) and period (prespray, 1st spray, 2nd spray). Period had no significant effect ($p=0.11$) while treatment was highly significant ($p=0.0001$). One way ANOVA of control river data alone showed no effect of period ($p=0.3642$). All 120 control samples were therefore pooled in further analyses. One way ANOVA showed a significant treatment effect ($p=0.0002$). Duncan's multiple range comparison of means (alpha=0.05) of controls to the 1st spray group (2% reduction) was not significant while comparison (alpha=0.05) of controls to the 2nd spray group was significant, a 9.2% reduction in activity being observed. (Table 2). There were no significant differences among AChE activities of aliquots of the same homogenate measured on different assay days (Table 3).

Fenitrothion was not detected in any of the stream water samples or in the control bog water. However, detectable levels of fenitrothion were observed in both bogs sampled in the spray block after the second spray application. Concentrations would be expected to be much higher in bogs than in streams which have a much greater dilution capacity. The levels of fenitrothion for the three replicate samples of bog water were 0, 0.0006, 0.0008 $\mu g\ mL^{-1}$ for the sprayed bog 2 km from the sprayed stream site and 0.001, 0.001 and 0.004 $\mu g\ mL^{-1}$ for the other sprayed bog sampled, 3 km from the sprayed stream site.

DISCUSSION

In this field study we measured the AChE activities in trout from a small unbuffered fenitrothion sprayed stream - essentially mimicking a worse case situation. Our results demonstrated limited inhibition of enzyme activity under such spray conditions.

Of what consequence is AChE reduction in fish? An AChE reduction of approximately 10% may indicate anti-cholinesterase activity, (Nicholson 1967; Holland et al. 1967) although Gibson et al. (1969) found this impractical. Weiss (1958) suggested that death occurs when brain AChE activity falls to 30-60% of normal. Ludke et al. (1975) suggested that for birds, inhibition exceeding 20% was indicative of exposure but that inhibition greater than 50% was required for diagnosing death. For birds, Zinkl et al. (1980) used the lower of the calculated values; the mean control activity less 2 standard deviations of the mean or the mean less 20% of the mean to judge whether an AChE activity was depressed. Lockhart

(1985) proposed that a reduction in brain cholinesterase activity to about 25% of pre-exposure levels (or a 75% reduction overall) be taken as significant in terms of potential toxicological effects in fish.

Determining how depression of enzyme activities will translate into adverse effects on a population of fish in a stream is a more difficult problem to address but one which may be approached by examining effects on fish exposed to various fenitrothion concentrations. Wildish and Lister (1973) suggested that a 70% reduction in AChE is sufficient to cause behavioural changes in trout. Post and Leasure (1974) found that a 75% reduction in AChE leads to a 70% reduction in swimming performance, a measure of their physical activity. By comparison, in our field studies enzyme activities were inhibited less than 10%. In general, it is often difficult to invoke real hazard when any putatively toxic effect in individual animals is small and transient.

In this study, we established AChE that enzyme activity was only slightly reduced in fish collected in a small unbuffered stream receiving two applications of fenitrothion in a normal forestry spray operation. This supports the hypothesis that present day forest spray practices which, in Newfoundland, require buffer zones, should have negligible toxicological effects on fish.

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REFERENCES

Cahill, M. 1987. Buffer zone regulations applied to forest insect control operations in Newfoundland and Labrador: An evolutionary perspective, p.2-3. In Buffer Zones: Their application to forest insect control operations. Proc. Buffer Zone Workshop, Eastern Spruce Budworm Council's Environmental Committee, Quebec City 16-17 April, 1986 124p ISBN 0-662-15732-X

Coppage, D. L., and E. Matthews. 1974. Short-term effects of organophosphate pesticides on cholinesterases of estuarine fishes and pink shrimp. Bull. Environ. Contam. Toxicol. 11(5): 483-488.

Coppage, D. L.,and T. E. Braidech. 1976. River pollution by anticholinesterase agents. Wat. Res. 10: 19-24.

Ellman, G. L., K. D. Courtney, V. Andres Jr. and R. M. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7: 88-95.

Fancey, L. L., J. F. Payne, M. A. Barnes, and R. M. McCubbin. 1987. Acetycholinesterase enzyme activity in fish sampled after the 1985 Newfoundland forest spray program for eastern hemlock looper using fenitrothion. Can. Tech. Rep. Fish. Aquat. Sci. 1579: iv + 10 p.

Gibson, J. R., J. L. Ludke, and D. E. Ferguson. 1969. Sources of error in the use of fish-brain acetylcholinesterase activity as a monitor for pollution. Bull. Environ. Contam. Toxicol. 4(1): 17-23.

Hill, E. F., and W. J. Fleming. 1982. Anticholinesterase poisoning of birds: Field monitoring and diagnosis of acute poisoning. Environ. Toxicol. Chem. 1: 27-38.

Hill, E. F. 1988. Brain cholinesterase activity of apparently normal wild birds. J. Wild. Dis. 24(1): 51-61.

Holland, H. T., D. L. Coppage, and P. A. Butler. 1967. Use of fish brain acetylcholinesterase to monitor pollution by organophosphorus pesticides. Bull. Environ. Contam. Toxicol. 2(3): 156-162.

Lockhart, W. L., D. A. Metner, and N. Grift. 1973. Biochemical and residue studies on rainbow trout (Salmo gairdneri) following field and laboratory exposures to fenitrothion. The Manitoba Entomol. 7: 26-36.

Lockhart, W. L., D. A. Metner, F. J. Ward, and G. M. Swanson. 1985. Population and cholinesterase responses in fish exposed to Malathion sprays. Pest. Biochem. Physiol. 24: 12-18.

Ludke, J. L., E. F. Hill, and M. P. Dieter. 1975. Cholinesterase(ChE) response and related mortality among birds fed ChE inhibitors. Arch. Environ. Contam. Toxicol. 3(1): 1-21.

Nicholson, H. P. 1967. Pesticide pollution control. Sci. 158: 871-876.

Post, G. and R. A. Leasure. 1974. Sublethal effect of Malathion to three salmonid species. Bull. Environ. Contam. Toxicol. 12(3): 312-319.

Weiss, C. M. 1958. The determination of cholinesterase in the brain tissue of three species of freshwater fish and its inactivation in vivo. Ecology 39(2): 194-199.

Weiss, C. M. 1961. Physiological effect of organic phosphorus insecticides on several species of fish. Trans. Am. Fish. Soc. 90(2): 143-152.

Wildish, D. J. and N. A. Lister. 1973. Biological effects of fenitrothion in the diet of brook trout. Bull. Environ. Contam. Toxicol. 10: 333-339.

Williams, A. K. and C. R. Sova. 1966. Acetylcholinesterase levels in brains of fish from polluted waters. Bull. Environ. Contam. Toxicol. 1(5): 198-204.

Zinkl, J. G., R. B. Robert, C. J. Henny, and D. J. Lenhart. 1980. Inhibition of brain cholinesterase activity in forest birds and squirrel exposed to aerially applied acephate. Bull. Environ. Contam. Toxicol. 24: 676-683.

Zinkl, J. G., P. J. Shea, R. J. Nakamoto, and J. Callman. 1987. Technical and biological considerations for the analysis of brain cholinesterase of rainbow trout. Trans. Am. Fish. Soc. 116: 570-573.

Table 1. Brain acetylcholinesterase activities (μmoles min^{-1} g^{-1} (wet weight) in trout sampled before and after spraying with fenithrothion. Samples were collected 72 h after the first spray application and 48 h after the 2nd spray application.

Prespray		1st Spray		2nd Spray	
Control River	Spray River	Control River	Spray River	Control River	Spray River
12.09	12.68	16.19	12.29	14.04	11.51
13.85	12.67	15.60	12.87	12.68	12.09
9.95	10.92	12.09	11.12	9.75	8.39
7.61	13.07	14.24	12.68	11.70	9.17
12.29	10.73	10.14	12.48	12.48	10.73
10.92	10.92	12.48	12.29	12.68	10.53
11.90	12.29	12.48	11.51	9.75	8.78
12.09	10.92	12.09	9.95	12.29	12.68
12.68	9.95	10.34	10.73	11.31	10.53
11.31	12.29	12.68	11.90	11.31	10.92
10.73	11.70	10.92	10.34	13.65	9.36
12.48	11.12	12.48	11.31	11.12	10.73
11.12	10.92	12.29	11.51	12.09	9.36
11.90	11.90	13.46	12.87	13.26	12.09
12.48	11.31	9.36	11.90	13.85	10.73
12.09	11.51	9.95	13.26	12.29	10.14
13.46	11.51	11.51	11.12	12.09	9.95
13.07	10.14	11.31	10.14	11.51	11.12
11.31	11.90	11.12	11.70	12.09	9.75
10.73	11.90	14.04	11.12	12.09	12.48
9.10	10.53	12.29	12.09	10.73	10.92
12.48	11.51	13.07	11.51	12.87	10.53
12.09	11.31	13.07	12.68	12.87	9.95
13.07	12.29	12.87	11.12	12.87	11.70
10.92	8.58	12.48	10.73	10.14	10.73
14.04	13.26	10.73	12.48	13.07	11.90
10.73	12.48	11.31	12.48	12.87	10.92
11.70	12.09	12.09	12.09	13.65	13.65
12.29	10.34	13.46	12.68	14.04	12.68
14.82	14.24	13.65	11.90	12.29	12.87

Table 2. AChE activity means (μmoles min^{-1} g^{-1}) and standard deviations.

Sample Treatment	n	AChE activity mean	S.D.	% Difference from the control mean
Controls	120	12.00	1.36	
1st Spray	30	11.76	0.87	2.00%
2nd Spray	30	10.90	1.28	9.17%

Table 3. Mean AChE activities (μmoles min^{-1} g^{-1}) of pooled trout brain homogenate done on different assay days to check for assay technique reliability (F = 1.7, df = 6, 13, n = 14, CV = 5.3%, p = 0.41).

Day	AChE activity
1	14.63
2	13.07
3	13.65
4	13.85
5	13.26
6	14.24
7	13.85

APPENDIX

Analytical protocol for the determination of AChE activity.

A. Each sample cuvette received:

1. 3.0 mL of 2.5×10^{-4} M 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), chromogen-buffer reagent (9.9 mg DTNB, 661mg Trizma HCl and 97mg Trizma base dissolved in 100 mL distilled water; pH 7.4. This solution is stable at 4°C in an amber bottle for 1-2 mo.)

2. 100 µL of 0.156 M acetylthiocholine iodide substrate (451.1 mg was made up to 10 mL with distilled water. This solution was made up daily.)

3. 20 µL of brain homogenate. Each homogenate was made at a ratio of 100 mg of brain to 1 mL of 0.05 M Trizma buffer, pH 8, (4.44 g Trizma HCl + 2.65 g Trizma base dissolved in 1000 mL of distilled water).

B. The solution in the sample cuvette was mixed quickly using a pasteur pipette, and placed in the spectrophotometer. The reference cuvette received chromogen and substrate but no homogenate. The sample cuvette was placed in the spectrophotometer and the change in absorbance was recorded for 2 to 3 min. at 405 nm, at 23°C.

C. The duplicate sample was assayed and the rate of change of absorbance per minute was determined from the chart recordings for both replicate samples. The average change in absorbance was multiplied by 130 to give the number of µmoles of acetylthiocholine iodide hydrolyzed per min per g of tissue (wet weight) (Table 1).

D. Enzyme Activity

Enzyme activity calculation (after Ellman et al. (1961) and Hill (1989), pers. comm.).

$$\frac{\Delta \text{ A/min} \times \text{Vol}_t \times 1000}{\text{E} \times \text{lightpath} \times \text{Vol}_s \times \text{tissue conc}} = \frac{\Delta \text{ A/min} \times 3.12 \times 1000}{13.3 \times 1 \times 0.02 \times 91}$$

$$= \Delta \text{ A/min} \times 128.9 \ (\approx 130)$$

$$= \text{µmoles min}^{-1} \text{ g}^{-1}$$

where: Δ A/min - Change in absorbance per min of DTNB at a wavelength of 405 nm.

Vol_t = Total assay volume (mL).

Vol_s = Sample homogenate volume (mL).

E = Absorbancy coefficient (13.3 cm^2 µmole^{-1})

Lightpath = cuvette width (1 cm)

Tissue conc. = Concentration of brain tissue (mg mL^{-1}).

Brain tissue is weighed and 10 times the brain weight in 0.05 M trizma buffer, pH 8, is added to give a ratio of 100 mg per 1.1 mL or 91 mg mL^{-1} (wet weight).

Calculation of AChE activity:

Δ A/Min x 130 - moles of acetylthiocholine iodide hydrolyzed per minute per gram of tissue (wet weight).

E. QUALITY ASSURANCE CHECKS

Brain tissue from four extra fish sampled from the control river after the second spray application was pooled and divided into aliquots in seven individual tubes of homogenate each of which was then run in duplicate on different assay days as a check on assay reliability (Table 3).

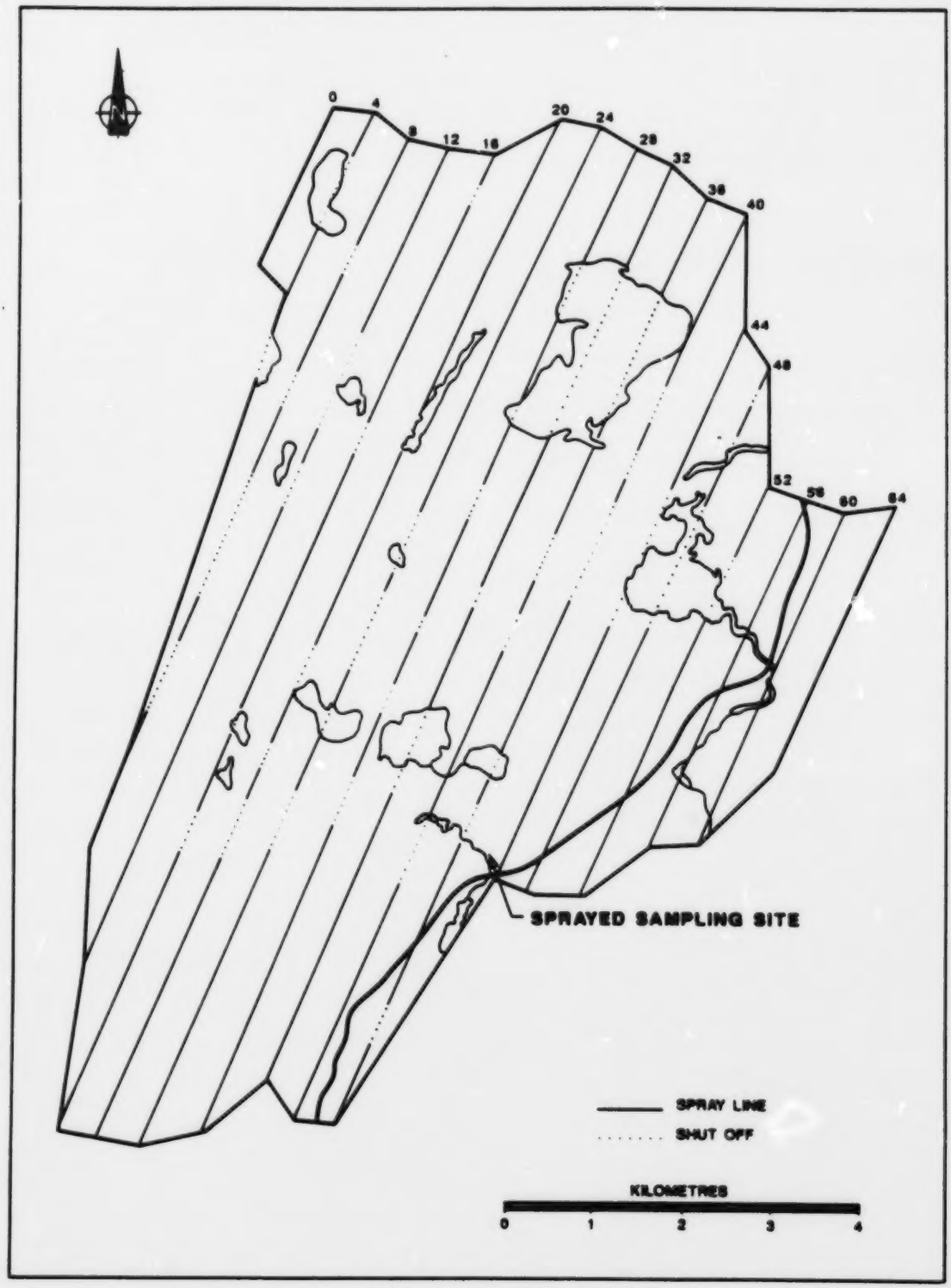

Fig. 1. A map showing flight lines in sprayed block 113.

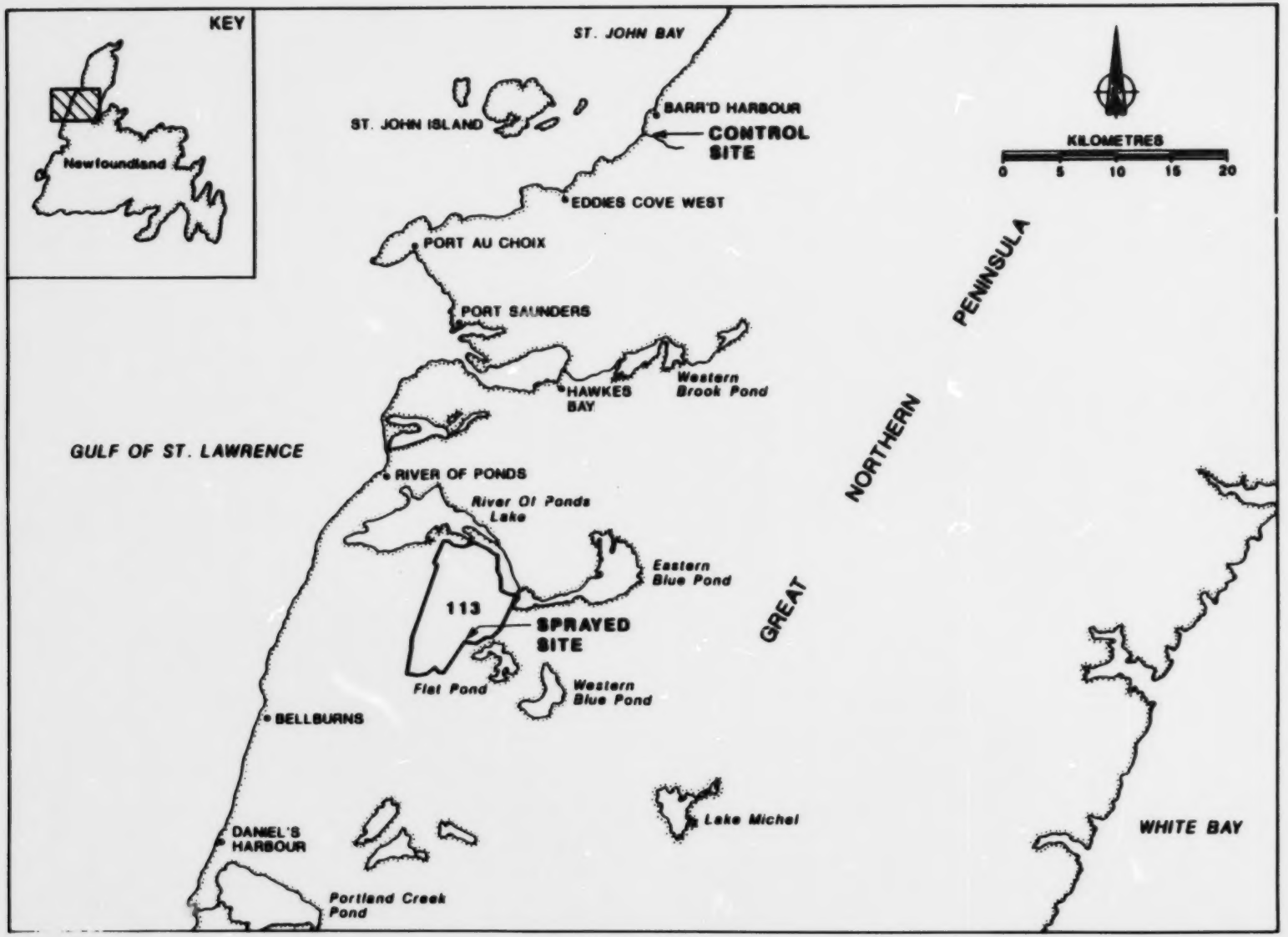

Fig. 2. A map of the Great Northern Peninsula showing 1988 sampling sites as denoted by the arrows.